Ins a, > c

Cobalamin Assay

BACKGROUND of The INVENTION

The present invention relates to assay methods for the determination of cobalamin or vitamin B_{12} in a body fluid and in particular to assay methods for the metabolically active pool of cobalamin.

Cobalamin or vitamin B_{12} is a water soluble vitamin which forms part of the vitamin B complex found in foods. The core molecule consists of a corrin ring of four pyrole units which surround the essential cobalt atom. Cobalamin is the only vitamin which cannot be synthesised by animals or plants and must be absorbed from food in the gut. It can however be stored in the liver. It is synthesised by micro-organisms, in particular by anaerobic bacteria and yeasts.

Cobalamin functions *in vivo* as a co-enzyme and cobalamin enzymes catalyse three types of reaction; (i) intra-molecular rearrangements, for example, the formation of succinyl CoA from L-methylmalonyl CoA, (ii) methylations, for example, the formation of methionine by methylation of homocysteine and (iii) reduction of ribonucleotides to deoxyribonucleotides in some micro-organisms. In mammals, only two enzymic reactions, those specifically mentioned in (i) and (ii) above are known to require cobalamin as a co-enzyme.

In the process of digestion, a salivary protein called haptocorrin, hereinafter referred to as HC (which is also referred to in the art as R-binder or transcobalamins I and III collectively) binds cobalamin in the upper gastrointestinal tract forming a complex which passes through the stomach. Pancreatic enzymes digest the cobalamin-haptocorrin (holo-HC) complex in the ileum, liberating cobalamin which is then bound to a protein called intrinsic factor, which is secreted by the gastric mucosa, to form a further complex. The cobalamin-intrinsic factor complex binds to a specific

receptor in the lining of the terminal ileum, whereupon
it is dissociated by a releasing factor and the
cobalamin transported actively across the membrane of
the ileum into the blood stream.

Cobalamin does not circulate in the body in a free
form in an appreciable amount. Probably 99% or so of
cobalamin is bound by one of the transcobalamin proteins
(TC I-III) or albumin.

The protein believed to be responsible for
transporting cobalamin to target tissues is
transcobalamin II (TC II), a critical trace protein
without which cobalamin cannot cross cell membranes.
Despite this important metabolic function only about 6-
25% of cobalamin in the serum is bound to TC II and most
is carried by HC. TC II is a single chain polypeptide
of 45 kDa found primarily in serum, seminal fluid and
cerebro-spinal fluid. Cobalamin bound TC II or holo-TC
II, attaches to specific receptors on cell membranes and
once bound, the holo-TC II complex is taken into cells
by pinocytosis.

TC II is synthesised by the liver, vascular
endothelium, enterocytes, macrophages and fibroblasts
and circulates predominantly as apo-TC II, i.e. lacking
bound cobalamin. It has a short half life of
approximately 90 minutes.

Less than a quarter of the total plasma cobalamin
is associated with TC II. The rest is bound to the
other transcobalamins or albumin as mentioned above.

Since cobalamin must be absorbed from food, any
conditions which result in impaired gastric function,
for example, gastroenteritis or conditions resulting in
gastric atrophy, or an inability to produce functional
haptocorrin, intrinsic factor, releasing factor, TC II
or TC II receptors, can result in impaired uptake of
cobalamin and resultant deficiency.

Certain population sub-groups, for example the
aged, pregnant women, patients with chronic or acute

gastrointestinal disease, those suffering from certain autoimmune diseases, those with a family history of pernicious anaemia and AIDS sufferers, are particularly prone to cobalamin deficiency.

The clinical manifestations of cobalamin deficiency are varied and numerous but primarily involve, anaemia, megaloblastic haematopoiesis and functional and structural disorders of the nervous system. Around 60% of individuals diagnosed as being deficient in cobalamin are anaemic, but in many, neurological symptoms are the only clinical signs observed. Around 10% of patients exhibit psychiatric symptoms and around 40% exhibit both neurological and psychiatric symptoms.

Early diagnosis of cobalamin deficiency is crucial to ensure a good prognosis for patients, since some of the manifestations of cobalamin deficiency, particularly the neuropsychiatric effects, are irreversible if not detected and alleviated by cobalamin therapy quickly.

It is desirable therefore to accurately assess the cobalamin level of an individual in an expedient and efficient manner, with a view to establishing whether or not the individual may be suffering from cobalamin deficiency.

Measurement of total plasma cobalamin ie. cobalamin (and cobalamin like substances) bound to any one of the transcobalamin (TC) proteins I, II and III, has been used in attempts to assess cobalamin deficiency. This technique results in a broad based concentration distribution within a population which is considered to be normal and hence produces a wide reference range. Within individuals however, the range of available cobalamin considered to be normal for that individual, is very narrow. It has been observed that although an individuals metabolically active cobalamin concentration has moved outside their own reference range, their total plasma cobalamin content remains within the range considered to be normal for the population. Under such

circumstances, cobalamin deficiency can go undetected.
Such an unreliable method is clearly undesirable and it
is well recognised that such serum or plasma cobalamin
measurements have low diagnostic sensitivity and
specificity.

Microbial assays involving micro-organisms
dependent upon cobalamin for growth, have been developed
and used in measuring plasma cobalamin concentration,
but in addition to the difficulty of estimating the
appropriate reference range, these methods require
extraction and conversion of the cobalamins which is
very time consuming, troublesome and wholly unsuited for
rapid laboratory screening.

Alternative methods for assessing cobalamin
deficiency involve measuring the accumulation of
metabolites in the plasma which require cobalamin for
their conversion. Plasma methylmalonate and plasma
homocysteine levels increase in cobalamin deficient
individuals (Chanarin, The megaloblastic anaemia;
London, Blackwell Scientific Publications, 1991)and make
good candidate molecules for correlation with vitamin B_{12}
deficiency. Methods based on homocysteine assessment
have been shown, however, to be complicated, impractical
and show poor specificity and sensitivity. Whilst
methods based on methylmalonate measurement are accurate
and reliable, they are cumbersome and require analysis
by combined gas-chromatography/mass-spectrometry and are
hence expensive and again unsuitable for routine
clinical screening (Nexø et al.(1994) Scand. J. Clin.
Lab. Invest. 54:61-76).

It has also been suggested that measurement of TC
II bound cobalamin as opposed to total plasma cobalamin
may provide a reliable clinical indictor of the
likelihood of cobalamin deficiency (Herbert et al.
(1990) Am. J. Hematol. 34:132-139; Wickramasinghe and
Fida (1993) J. Clin. Pathol. 46:537-539; US Patent
4680273). However, such efforts to determine holo-TC II

concentratration have to date been mostly indirect,
estimating holo-TC II concentration as the difference
between total plasma cobalamin and the cobalamin
concentration of TC II depleted plasma.

Such TC II depletion may be accomplished by
adsorption to ammonium sulphate (Carmel (1974) Am. J.
Clin. Pathol. 62:367-372), microsilica (Herzlich &
Hubert (1988) Lab. Invest. 58:332-337; Wickramasinghe &
Fida (1993) J. Clin. Pathol. 46:537-539), microfine
glass (Vu et al. (1993) Am. J. Hematol. 42:202-211) or
immobilized anti-TC II polyclonal antibodies (Lindemans
et al. (1983) Clin. Chim. Acta 132:53-61). The
concentration of cobalamin in total plasma and the
depleted fraction is performed by methods well known in
the art such as radio or enzyme immunoassay techniques.
These methods are unsuitable for routine screening
automated or not automated because they are complex and
time consuming and because the low degree of specificity
of the adsorptive materials used results in insufficient
separation of holo-TC II and holo-HC resulting in an
overestimation of holo-TC II. Lot-to-lot variation of
the adsorptive material introduces further errors and
most importantly, the subtraction of one large volume
from another large volume results in unacceptable
inaccuracies and unreliability.

The other attempts to assess TC II have involved
separating TC II from other serum components, including
the TC I and TC III, using its lipophilicity. Thus
Kapel et al. (1988) Clin. Chim. Acta 172:297-310,
Benhayoun et al. (1993) Acta Haematol. 89:195-199 and
Toft et al.(1994) Scand. J. Clin. Lab. Invest. 54:62
disclose methods for separating TC II from other
transcobalamins using heparin sepharose, silica gel or
cellulose respectively. These methods however suffer
from the same disadvantages as the indirect methods
since they rely on the same adsorptive materials. Also,
the low plasma concentration of holo-TC II renders these

methods unsuitable for combination with existing methods
of cobalamin quantification. The normal range of holo-
TC II is 35-160 pM and values below 35 pM would
generally be considered as indicative of cobalamin
deficiency. The reported analytical sensitivity of most
routine methods for plasma cobalamin is about 40 pM but
in practice it is often much higher, typically around 90
pM. Hence, normal plasma levels of holo-TC II are below
or near the sensitivity limit of the routine methods for
cobalamin quantification.

Possibly the most accurate method currently
recognised for determining TC II bound cobalamin
involves adsorbing TC II to silica and then assaying the
bound fraction for cobalamin content using either an
immunoassay as described for example by Kuemmerle et al.
(1992) Clin. Chem. 38/10: 2073-2077, or a
microbiological assay, the latter apparently producing
the best results. This method requires an entire
working day to perform only twenty assays. It is very
expensive and impractical and poorly suited to routine
clinical diagnostic laboratory investigations.

Thus, there exists a great need for improved
methods of assessing the level of metabolically active
cobalamin in a body fluid, with a view to correlating
the cobalamin level with the likelihood of cobalamin
deficiency, which are amenable to routine clinical
diagnostic application.

Summary of The Invention

Thus, according to a first aspect, the present
invention provides an assay method for the determination
of transcobalamin II bound cobalamin in a body sample,
comprising contacting a cell free sample of a body fluid
with an immobilised or immobilizable specific binding
ligand for TC II or holo TC II, separating a ligand
bound fraction from a non-ligand bound fraction and
measuring the holo-TC II or TC-II bound cobalamin
content therein.

Detailed Description of The Invention

By a specific binding ligand is meant one which

binds to TC II (ie. apo-TC II and holo-TC II) or holo TC
II by virtue of its specific chemical structure or
conformation and not simply by virtue of an overall
physico-chemical property (such as lipophilicity) which
may be common to many components of a body fluid sample.
The binding affinity and specificity of the ligands
suitable for use in the method preferably allow for a 3-
fold, more preferably a 5-fold and even more preferably
a 10-fold and most preferably a greater than 10-fold
concentration of the TC II or holo-TC II in the sample
and hence such a method is able to give accurate and
reliable values for holo-TC II in the lower normal range
of holo-TC II and also in the sub-normal range. Such a
separation and concentration of the target molecules is
not possible with currently existing methods which are
unable to efficiently separate TC II or holo-TC II from
HC or holo-HC. The ability to concentrate the TC II or
holo-TC II at least 3-fold allows the use of automated
analytical equipment in the performance assay. Without
such a concentrating step, the amount of analyte present
in a sample is likely to be below the lower detection
limit. Such automated analytical equipment typically
requires between 40 and 100 μl of sample in a total
volume of typically 150 μl or greater for its operation.
Thus, a low concentration analyte is diluted even
further for analysis. By using the assay method of the
invention however, the analyte is concentrated at least
3-fold. Since using such automated analytical equipment
typically involves a control range of 100-700 pM with a
lower sensitivity limit of about 40 pM but a practical
lower limit of around 90 pM, a 5-fold concentration
facilitates the measurement of holo-TC II at down to 18
pM and 10-fold concentration facilitates measurement
down to 9 pM holo-TC II. Since the present invention
facilitates concentration of greater than 10-fold it
will be easily understood by the skilled person the
extent to which sensitivity is enhanced making it a very

powerful technique indeed.

The ability to concentrate the analyte so that it is amenable to analysis by such automated procedures is an important advantage of the present assay method.

Preferably from a 600 μl starting sample of body fluid, a volume of up to 150 μl, more preferably up to 100 μl and most preferably less than 60μl will be generated which may then be analysed, optionally using automated procedures.

Any TC II specific binding ligand may be used in the method of the invention as either a capturing, concentrating and separating ligand or a detecting ligand, and depending on the specific embodiment of the invention may bind to TC II in both the apo and holo form or it may bind specifically or preferentially to the holo-form. The TC II binding ligand will preferably exhibit a high degree of selectivity and specificity towards TC II and will exhibit low or more preferably, essentially no affinity towards other TC proteins, ie. TC I or III, in either apo or holo form, or any other cobalamin-binding protein. If the TC II binding ligand is a holo-TC II specific binding ligand, the same properties are exhibited with the additional requirement that low or preferably no affinity is shown towards apo-TC II.

The TC II or holo-TC II binding ligand will generally be either an antibody, an antibody fragment or a compound with an affinity for TC II or holo-TC II respectively such as a cell surface receptor, a polypeptide, an oligopeptide, a small organic chemical, etc. Other binding ligands may be a specific binder selected from a combinatorial chemistry or phage display library or a specifically binding sequence of DNA or RNA.

If the binding ligand is an antibody it may be polyclonal but will preferably be monoclonal. Monoclonal antibodies can be generated with much greater

specificity and uniformity than polyclonal antibodies
and this reduces cross-reactivity with other components
of the body fluid, in particular other transcobalamins
and where appropriate, the alternative conformation ie.
the apo form of the target analyte. The uniformity and
reproducibility offered by monoclonal antibodies
relative to polyclonal antibodies ensures a greater
accuracy which is vital for an assay wherein the analyte
is in such low concentration. Alternatively, it may be
an antibody fragment for example F(ab), F(ab')$_2$ or F(v)
fragment. The antibodies or antibody fragments may be
monovalent or divalent and may be produced by hybridoma
technology or be of synthetic origin, and generated by
recombinant DNA technology or chemical synthesis.
Single chain antibodies or other antibody derivatives or
mimics could for example be used. The antibody may be
directed or raised against any epitope, component or
structure of the TC II or holo-TC II protein as
appropriate.

Suitable antibodies for use as binding ligands in
the present invention are disclosed for example by
Quadros *et al.* (1996) Biochem. Biophys. Res. Commun.
222:149-154; McLean *et al.* (1997)Blood 89(1):235-242.

Receptor molecules capable of binding the apo and
holo forms of TC II or preferentially or specifically
binding the holo-TC II form may similarly be used.
Suitable receptors are cell surface or membrane bound TC
II or holo-TC II receptors and cross species reactivity
properties mean that such receptor molecules from any
mammalian species may be used, although preferably the
origin of such receptors is human or bovine. Although
the receptor molecules are preferably isolated in a
relatively purified form, the use of cell membranes or
mixed membrane fractions comprising the receptors
preferably in concentrated form is also contemplated.
Such receptors or receptor containing membranes or
membrane fractions may advantageously be isolated from

kidney, placenta or tumor cells. Deposit cells should
not generally be used as a source of such receptors.
Deposit cells may however be used as a source of
haptocorrin receptors.

A membrane fraction enriched in TC-II receptor can
be obtained as described by Seligman et al., J. Biol
Chem 253:1766-1772 (1978) and Nexø et al., Biochem
Biophys Act 628: 190-200 (1980). Essentially, tissue,
e.g. human placenta or rabbit liver, is cut into small
pieces and homogenized in tris buffer, pH 7.4 containing
0.15M NaCl and 10 mM EDTA, followed by centrifugation at
25,000 x g for 30 minutes. To remove residual blood the
homogenization/centrifugation is repeated at least one
time. This membrane fraction is further extracted with
detergent, e.g. Ammonyx-LO, Triton X-100 or Chapso, and
clarified by centrifucation at 100,000 x g for 30
minutes at 4°C. The supernatant is used as the source
for TC-II receptor.

An example of a suitable receptor molecule which
preferentially binds the holo-TC II complex is the 62
kDa single chain glycoprotein which exists as a
noncovalent homodimer and is found on the cell surface
of all tissues (Rothenberg & Quadros (1996) Balliere's
Clinical Haematology 8(3) 499-514; WO 96/085150). A
further protein suitable for use in the assay method of
the invention is gp300, a 600 kDa endocytosis mediating
membrane protein which is expressed in absorptive
epithelial cells for example, cells of the renal
proximal tubule as disclosed and described by Moestrup
et al., (1996) Proceedings National Academy Science 93:
8612-8617). This receptor is an LDL receptor and binds
both the apo and holo forms of TC II.

Where a cell surface receptor is used, it can be
immobilized by conjugation to a surface, e.g. of a bead
or sheet, or alternatively a cell membrane fraction
containing the receptors may be formed into vesicles or
sheets displaying the receptors.

- 11 -

Where the ligand is immobilized, this may for
example be on the surface of a solid, e.g. a filter film
or sheet or the lumen of a tube, however immobilization
on particles, e.g. microspheres such as those produced
by Dyno Industrier ASA, Norway, is especially preferred.
Such particles may be porous or non-porous and if
desired may be provided with means which enable them to
be collected, e.g. by inclusion with the particles of
magnetically responsive material, for example
superparamagnetic iron oxide crystals. Such
magnetically responsive microbeads are available from
Dyno Industrier ASA as well as from Dynal AS, Norway,
Bang Particles, USA and Prolabo, France. Where the
ligand is to be immobilizable as opposed to immobilized,
this may be achieved by coupling the ligand to a member
of a specific binding pair (e.g. biotin/streptavidin)
and using the other member of the binding pair,
optionally bound to a substrate, to agglomerate or
otherwise immobilize the ligand or the ligand:TC II or
ligand: holo-TC II complexes, prior to separation of the
ligand bound fraction from the non bound fraction in the
performance of the method of the invention.

It is well known in the art to immobilise affinity
molecules e.g. antibodies and antibody fragments for
separation purposes, for example by binding or coupling
the ligands, optionally by means of a linker, to any of
the well known solid supports or matrices which are
currently widely used or proposed for separation or
immobilisation on columns and any known method in the
art could be used. Such solid phases may take the form
of particles, sheets, gels, filters, membranes, fibres
or capillaries or microtitre strips, tubes or plates of
wells etc and conveniently may be made of glass, silica,
latex or a polymeric material. Techniques for binding
the ligand to the solid support are also extremely well
known and widely described in the literature.

Coupling of the ligand to a substrate or to one

member of a specific binding pair may be achieved using
conventional techniques.

The method of the invention, if the assay is
performed as a competitive binding assay, will generally
involve addition to the sample of a labelled compound
which competes for binding to the ligand. Generally, it
will be preferred to use labelled holo-TC II in this
regard. The label used may be any label which can be
determined directly or indirectly, e.g. a chromophore
(which term is used to include fluorophores), a
radiolabel (generally covalently bound or chelate-
bound), an enzyme or enzyme substrate, a magnetic label,
etc.

A preferred embodiment, the method of the present
invention involves contacting an immobilised or an
immobilisable TC II or holo-TC II binding ligand with
the sample under investigation;

separating a ligand-bound fraction from a non-
ligand-bound fraction;

dissociating bound cobalamin from the holo-TC II
molecules in the bound fraction and determining the
concentration of cobalamin released, preferably with
dissociation being so affected that the concentration of
the released cobalamin is at least 3 times, preferably 5
times and even more preferably 10 times greater than the
concentrations of holo-TC II in the initial sample.

The essence of this aspect of the invention is the
separation and concentration of TC II or holo-TC II
which allows standard methods of cobalamin determination
to be usefully employed in the method. As indicated
above, in the absence of such a concentration step state
of the art methods are not suitable for cobalamin
determination since it exists at such a low
concentration. Any appropriate means for releasing
cobalamin from the holo-TC II may be used but
conveniently heat or changing the pH of the surrounding
medium may be used in this regard. The different

- 13 -

cobalimin forms may be converted to the less light
sensitive cyanocobalamin by treatment with KCN. Any
appropriate means for free cobalamin determination may
be employed in the method of the invention for example a
competition assay performed by contacting an immobilised
binding partner for cobalamin with the dissociated
cobalamin of the sample in the presence of labelled
ligand which competes with the isolated cobalamin for
binding to the immobilised binding partners. A method
as described by Kuemmerk et al. (1992) Clin. Chem
38:2073-2077 for example would be appropriate. An
alternative means for determining free cobalamin is
described in US Patent No. 5451508 and involves a
immunoassay technique.

In this embodiment of the invention it is preferred
that the binding ligands for TC II are immobilised and
bind to both holo and apo-TC II.

In an alternative preferred embodiment, the assay
method of the present invention comprises contacting a
solid support having immobilised thereon a TC II or
holo-TC II binding ligand, with the sample under
investigation and also with a non-immobilised ligand,

wherein said immobilised ligand is capable of
binding to TC II or holo-TC II, to said non-immobilised
ligand or to complexes of said TC II or holo-TC II and
said non-immobilised ligand, and said non-immobilised
ligand is capable of binding to at least one of said
immobilised ligand, TC II or holo-TC II and complexes of
said immobilised ligand and TC II or holo-TC II;

wherein if said assay method is a sandwich assay,
at least one of said ligands is specific for holo-TC II
and if said assay is a competition assay said
immobilised ligand is specific for holo-TC II and
competitors thereof;

whereby the proportion of said immobilised ligand
bound by TC II or holo-TC II, by said non-immobilised
ligand or by complexes of said non-immobilised ligand

and TC II or holo-TC II is dependent on the amount of
holo-TC II present in said sample, and,

said non-immobilised ligand is capable of
generating a directly or indirectly detectable signal
when bound or when unbound;

separating a bound fraction from a non-bound
fraction; and

directly or indirectly determining the non-
immobilised ligand bound to the immobilised ligand (the
bound fraction) or non-bound and in solution (the non-
bound fraction);

where the contacting of the sample and said non-
immobilised ligand with the solid support may be
performed separately, simultaneously or sequentially,
and if performed separately or sequentially, they can be
contacted in either order.

In essence therefore, the alternative preferred
embodiment of the method of the invention involves
determining the non-immobilised ligand which has either
directly or indirectly bound or alternatively failed to
bind directly or indirectly to the immobilised ligand.
Where the non-immobilised ligand competes for binding to
the immobilised ligand with the holo-TC II, a high level
of unbound non-immobilized ligand is indicative of a
high concentration of holo-TC II in the sample and a low
level of unbound non-immobilized ligand is indicative of
a low concentration of holo-TC II in the sample. Where
the non-immobilised ligand binds to the TC II or holo-TC
II which is bound in turn to the immobilised ligand,
then a high level of bound non-immobilized ligand is
indicative of a high concentration of holo-TC II in the
sample and a low level of bound non-immobilized ligand
is indicative of a low concentration of holo-TC II in
the sample.

In the method of the present invention therefore,
the metabolically active pool of cobalamin is determined
by measuring the holo-TC II complex or measuring the

amount of cobalamin bound to TC II molecules, present in
a body sample which contains a mixture of both apo and
holo-TC II and apo and holo-HC (haptocorrin or TC I and
III).

A preliminary separation step may be carried out
using immobilised cobalamin or an analogue or fragment
thereof which selectively binds to the apo-forms of both
TC II and haptocorrin (HC). In such a preliminary step,
the apo forms of the TC II and HC proteins are bound by
the immobilised cobalamin, analogue or fragment thereof
and separated from the holo-TC II and holo-HC complexes.

In this case, analysis of the separated holo-forms
of TC II then takes place according to any embodiment of
the invention as described above but clearly it is most
useful where determination of the holo-TC II complex
takes place by other than dissociation of the complex
and subsequent determination of the released cobalamin.
It will be apparent to the skilled person that if such a
preliminary step precedes the above-mentioned
alternative preferred embodiment, the requirement for at
least one ligand in a sandwich assay to be specific for
holo-TC II as opposed to TC II and the requirement for
the immobilised ligand in a competition assay to be
specific for holo-TC II and competitors thereof as
opposed to TC II is no longer relevant since the apo-
form of TC II has been captured and is no longer
available. Thus, the immobilised or non-immobilised
ligands may be specific for holo-TC II or TC II or
competitors thereof.

The immobilised cobalamin should not exhibit any
significant tendency to become dissociated from its
support since this could result in binding to apo-TC II
and transformation thereof to holo-TC II. Biotinylated
cobalamin binds in a very stable manner to a solid
surface with avadin/streptavidin bound thereto, and
tethering cobalamin to a support in this manner is
convenient for the performance of this step. In fact,

- 16 -

when biotinylated cobalamin is used, can be added to the sample under analysis in a non-immobilised form where it binds to the apo-forms of TC II and HC. The sample may then be contacted with a solid surface having a binding partner for the biotin example, avidin, immobilised thereon. A complex of avadin-biotinylated cobalamin-TC II then forms which can be easily isolated from the sample.

In one embodiment of the invention wherein this preliminary separation step has taken place, the holo-TC II pool is subsequently determined by contacting the sample with an immobilised TC II ligand which captures the holo-TC II complex leaving the haptocorrin in solution and then contacting the immobilised holo-TC II with a second TC II ligand which is labelled and thus detectable. Examples of suitable TC II binding ligands would be non-overlapping monoclonal antibodies or indeed a polyclonal antibody specific for TC II would be suitable in this embodiment for both capture and detection since different epitopes on the TC II molecules are recognised.

In another embodiment of the invention wherein this preliminary separation step has taken place, the holo-TC II molecules compete with the labelled non-immobilised ligand for binding to the immobilised ligand therefor and the amount of holo-TC II is calculated in relation to the amount of labelled non-immobilised ligand bound or not bound to the immobilised ligand.

In a preferred embodiment, in the preliminary separation step, the binding of apo TC II and apo HC to cobalamin, analogues or fragments thereof takes place at a site or in such a manner which inhibits subsequent recognition and binding of the immobilised cobalamin bound TC II by the non-immobilised ligand or binding partner for TC II. In this embodiment there is no need to isolate the holo-TC II and holo-HC from the bound apo forms before performing the assay of the invention. In

this embodiment, the site against which the non-
immobilised binding partner or ligand is directed is
very important and should be an epitope on TC II which
becomes masked or shielded or otherwise unavailable for
binding when the apo TC II and apo-HC becomes
immobilised on the cobalamin, analogue or fragment
thereof.

Whether the initial separation step involves using
a binding ligand for TC II or it is preceded by a
preliminary step using immobilised cobalamin, analogues
or fragments thereof, total separation, ie. separation
of all the apo and holo TC II proteins, from the sample,
is not a requirement of the method and it suffices that
a fraction is separated from the sample which comprises
at least a portion of the desired 'TC II subset of
proteins'.

Thus, in certain embodiments, the binding partners
or ligands and binding conditions may be selected so as
to achieve separation of substantially all of the
selected "subset". In this case the non-bound fraction
may be regarded as being substantially free of TC II or
holo-TC II proteins, i.e. being at least 80%, 90% or 95%
free of either TC II or holo-TC II depending upon which
component is selected as the basis of fractionation.

In alternative embodiments, the binding partner and
conditions may be selected so that only a part of the TC
II proteins in the sample are separated into a fraction.
In this case, account should be taken of the partial
separation in constructing a standard calibration curve
for the assay. In this regard, the generation of a
standard calibration curve against which the
concentration of the holo-TC II detected can be
determined uses standard techniques well known in the
art.

Thus, if in a fractionation step, a TC II binding
ligand is used, at least a portion of the TC II bound
cobalamin will be located in the bound fraction and any

cobalamin in the sample bound to molecules other than TC
II, for example, HC or albumin will be in the non-bound
(ie. non-separated fraction or fractions). TC II in the
bound fraction may be in either apo and holo form and
may be complexed by cobalamin like substances or
analogues in addition to cobalamin.

If desired, the method of the invention may involve
a further preliminary separation step in which the
sample is contacted with an immobilized or immobilizable
specific binding ligand for haptocorrin (in apo and holo
forms or in holo form alone). In this way any
contribution to errors in the TC II-bound cobalamin
determination deriving from holo-HC may be reduced and
specific binding ligands for TC II or holo-TC II may be
used which have some binding affinity for haptocorrins.

Otherwise, as indicated above, since the serum
concentration of TC II both in apo and holo form is very
low, ligands or binding partners of particularly high
specificity and affinity are necessary for the operation
of the invention. Also, the affinity constants required
of the ligands of the present invention depend upon
whether the ligand is specific for TC II or holo-TC II
and also its utilization as either a capture or
detection ligand, since the serum concentration of TC II
is about 0.5-1 nM but the concentration of holo-TC II
only 35-160 pM. Thus, for a TC II capturing ligand an
affinity constant of at least $10^9 M^{-1}$, preferably greater
than $2 \times 10^9 M^{-1}$ and more preferably $10^{10} M^{-1}$ is desirable.
For a holo-TC II capturing ligand, an affinity constant
of at least $10^{10} M^{-1}$ is desirable, preferably $2 \times 10^{10} M^{-1}$,
more preferably greater than $2 \times 10^{10} M^{-1}$ and most preferably
greater than $10^{11} M^{-1}$. Clearly the affinity constant
required of a detecting binder may be less than that of
a capturing ligand due to the concentration effect of
the assay.

The degree of cross reactivity of a holo-TC II or
TC II binding ligand with HC should preferably be less

than 1%, more preferably between 0.1% and 1% and most
preferably less than 0.1%. Similarly, a holo-TC II
binding ligand should preferably not exhibit a degree of
cross reactivity with apo-TC II in excess of 1%, more
preferably between 0.1% and 1% and most preferably the
cross-reactivity should be less than 0.1%.

By using such high affinity ligands their function
is extended beyond that of simply being capture and/or
detection ligands and they play a vital role in being
able to separate and concentrate TC II protein from the
sample for analysis. The binding ligands for TC II or
holo-TC II should preferably concentrate the ligand by
at least 3-fold, more preferably 5-fold, and even more
preferably by at least 10-fold.

In a further embodiment of the assay technique more
akin to a displacement assay than a competition assay,
the sample comprising the holo-TC II complex is
contacted with a solid phase to which a labelled ligand
recognising the same binding sites on the immobilised
ligands as holo-TC II, is bound. The holo-TC II in the
sample competes with the bound labelled ligand for the
sites such that after equilibration of the system, there
is a directly proportional relationship between the
amount of labelled ligand displaced from the solid
support and detectable in solution and the amount of
holo-TC II present in the original sample. The labelled
ligand may be detected directly or indirectly and may be
determined as the amount of labelled ligand bound or not
bound to the solid support as appropriate. In effect,
in this embodiment, the non-immobilised ligand referred
to earlier is bound to the immobilised ligand before
application of the sample but such binding should not be
construed to mean that the ligand is in any way
immobilised.

A further preferred embodiment involves contacting
a holo-TC II containing sample with a solid support
having holo-TC II immobilised thereon and a labelled un-

immobilised holo-TC II specific binder. The free holo-
TC II in the sample and the immobilised holo-TC II
compete for binding with the labelled non-immobilised
ligand and determination of the labelled ligand bound
the solid phase or remaining in solution allows
calculation of the holo-TC II concentration. In a
particularly preferred embodiment of the present
invention the labelled non-immobilised holo-TC II
binding ligand is an antibody.

In yet another embodiment, the sample comprising
the analyte of interest is contacted with labelled holo-
TC II and immobilised ligand. The labelled and non-
labelled holo-TC II compete for binding to the
immobilised ligand and after equilibrium is reached, the
amount of labelled holo-TC II bound to the immobilised
ligand is indirectly proportional to the amount of holo-
TC II in the sample of interest. Again, the labelled
holo-TC II may be detected directly or indirectly and
may be determined as the amount of labelled holo-TC II
bound or not bound to the solid support as appropriate.

In a further embodiment of the present invention, a
non-immobilised but immobilizable ligand specific for
the holo-TC II complex, (e.g. a ligand conjugated to
biotin or another member of a specific binding pair) is
contacted with the sample to form a holo-TC II/non-
immobilised ligand complex. The ligand/holo-TC II
complex may then be precipitated from solution using
known means and isolated for analysis.

In yet another embodiment of the invention, two
labelled binding partners are added to the sample,
optionally after a preliminary step such as depleting
the sample of the apo forms of TC II and HC. The
binding partners in this case are labelled holo-TC II or
an analogue or fragment thereof and a labelled binding
partner therefor, for example a labelled antibody. In
this embodiment a detectable signal is only generated
from the labels when they are in close proximity to each

other ie. when the two labelled binding partners become
bound to each other. Thus, on addition to the sample,
the labelled binding partner for holo-TC II may bind
either to unlabelled holo-TC II present in the sample in
which case no detectable signal is generated or to
labelled holo-TC II or an analogue, fragment or variant
thereof which binds the labelled TC II binding partner,
in which case a detectable signal is generated.
Labelling agents such as those of Amersham scintillation
proximity assays as described in US Patent No. 4568649
are suitable for incorporation into such an embodiment
and the high sensitivity of this procedure is well
suited to an assay wherein the analyte exists at such a
low concentration. Thus for example, one member of the
binding pair could be labelled with a β-emitting nuclide
such as I^{125} or H^3 and the other member is labelled with a
suitable scintillant molecule. The emitted β particle
loses its energy in its aqueous surroundings unless the
scintillant is within 1.5 μm and thus requires both
labelled partners to be bound to each other for a signal
to be detectable. The probability of two labelled
partners binding each other is determined by the
concentration of holo-TC II present in the sample such
that the greater the signal generated, the lower the
concentration of holo-TC II in the sample.

    As used herein, the terms "determining" or
"assessing" include both quantitation in the sense of
obtaining an absolute value for the amount or
concentration of holo-TC II or TC II bound cobalamin in
the sample, and also semi-quantitative and qualitative
assessments or determinations. An index, ratio,
percentage or similar indication of the level or amount
of TC II bound cobalamin, for example relative to total
cobalamin, may be obtained.

    The body sample used in the assay method of the
invention may be any cobalamin containing sample e.g. a
body fluid or tissue sample, or a suspension etc.

Preferably, however the sample will be a body fluid for
example, seminal fluid, cerebro-spinal fluid or amniotic
fluid, but will generally be a blood derived sample.
When this is the case, as the sample used for analysis
is preferably cell-free, either serum or plasma may be
used. The sample may be treated prior to being used in
the assay method of the invention, for example it may be
diluted by adding a buffer or other aqueous medium and
may be stored or preserved for example by chilling or
freezing prior to analysis.

In the practise of the invention, where a bound
fraction is separated from an unbound fraction this may
be performed by any suitable means, for example,
precipitation, centrifugation, filtration,
chromatographic methods etc.

For the avoidance of doubt, the term "cobalamin" is
used herein synonymously with "vitamin B_{12}" and includes
all forms of vitamin B_{12} (cyanocobalamin; 5-6-dimethyl-
benzimidazolyl cyanocobamide; methylcobalamine; 5'-
deoxyadenosylcobalamin) as may occur and be
metabolically active (when appropriately presented) in
the body.

As indicated above, in the method of the invention,
the determination of the TC-bound cobalamin may be
performed by detecting a ligand bound to the separated
holo-TC II, or by performing a competition assay using a
labelled competitor to holo-TC II whereby the holo-TC II
competes with the labelled competitor for binding to a
binding ligand therefor, or by detecting cobalamin
released from separated holo-TC II.

The detectable ligand may conveniently be labelled
by any conventional means, for example with a signal
forming label which may be determined by e.g.
luminescence, chemiluminescence, colorimetric
assessment, fluorescence, radioactivity or by enzymic
activity. In effect, any signal forming label known in
the art may be used in the method of the invention.

By means of example only, some suitable examples of coloured or fluorescent compounds which may be used to label a binding ligand detectably in the present invention are anthraquinones, azodyes, azine dyes such as oxazines and thiazines, triazines, naturally occurring pigments such as porphyrins, phycobiliproteins, including phycoerythins and phycoquanins, chlorophylls and their analogues and derivatives, carotenoids, acrinidines, xanthenes including fluoresceins and rhodamines, indigo-dyes, thioxanthenes, coumarins, polymethines including di and tri arylmethines and derivatives thereof of phthalocyanines and metal phtalocyanines.

Similarly, a wide range of radioactive compounds may be used as the signal forming label part of the reagent used in this invention, among them Iodine-125-labelled compounds.

Alternatively, the binding ligands may be conjugated to natural or synthetic compounds which can produce a chemiluminescent signal which may be assayed in known manner (Cormier, M.J. et al.; Chemiluminescence and Bioluminescence, Plenum Press, New York 1973). Suitable chemiluminescent compounds include luciferin, oxalic esters, 1,2-dioxethane, luminol or derivatives thereof, but are not limited to these. If appropriate, hydrogen peroxide, enzymes e.g. luciferase, or other chemicals may be used to produce the chemiluminescent signal from the signal-producing molecules used.

Strongly-anionic signal forming molecules may not be preferred for use in the method of the invention, since they have a tendency to bind to serum proteins such as human serum albumin (HSA) which may be present in the sample. Particularly suitable examples which may be used are fluorescein isothiocyante, Rhodamine B or N-(resorufin-4-carbonyl)piperidine-4-carboxylic acid-N-hydroxysuccinimide-ester) or resos.

In accordance with one embodiment of the method of

- 24 -

the invention, a fraction comprising at least a portion
of the TC II may be separated from a body fluid sample
by reacting the body fluid with a binding ligand
specific for TC II and then separating the TC II bound
fraction from the rest of the sample, thereby isolating
and concentrating the target analyte therein. In one
embodiment of the invention, a detectable binding ligand
directed against holo-TC II may be used to determine the
TC II-cobalamin complex present in the separated bound
fraction. The holo-TC II binding ligand may be
contacted with the sample under investigation prior to,
simultaneously with or after contact with the TC II
binding ligand and before or after separation of the TC
II bound fraction from the rest of the sample.

        Correspondingly, a cobalamin containing fraction
(e.g. comprising holo-TC II and holo-HC) may be
separated from the rest of the sample by reacting the
body fluid under investigation with an immobilised or
tethered cobalamin or an analogue or fragment thereof
which binds apo-TC II and apo-HC and separating from the
bound fraction the rest of the sample which comprises
holo-TC II and holo-HC. A detectable TC II binding
ligand can then used to detect the TC II-cobalamin
complex present in the separated fraction. The
detectable TC II binding ligand may be contacted with
the sample under study prior to, simultaneously with or
after contact with the bound cobalamin and before or
after separation of the bound fraction from the rest of
the sample.

        In some embodiments, immobilised ligands for one or
the other components of the apo and/or holo TC II/HC
complexes may be arranged in a column. The body fluid
containing the TC II cobalamin complex may be applied to
the column and contacted with the binding ligand(s).

        The column may be flushed through or washed and an
eluent used to allow, if necessary, release of a bound
fraction from the column and facilitate its collection.

If the contents of the unbound fraction are or are also
to be analysed with respect to other constituents, the
column should be washed with a buffer or medium
administered using a calibrated micropipette to ensure a
precise known volume is administered and collected. The
volume administered is preferably within 3% of the
desired (ie. calibration) volume, more preferably within
1 or 2%. Likewise, if the fraction to be analysed is to
be released from the column prior to detection, the
eluant used to release the complex should be
administered using a calibrated micropipette.

In an alternative embodiment, the binding ligands
used to separate a fraction of a sample may be
immobilised on a particulate solid phase support, for
example latex or polymer beads. To aid manipulation and
separation, magnetic beads may be used and indeed this
is a preferred embodiment of the present invention. the
term "magnetic" as used herein means that the support is
capable of having a magnetic moment imparted to it when
placed in a magnetic field. In other words, a support
comprising magnetic particles may readily be removed by
magnetic aggregation, which provides a quick, simple and
efficient way of separating the fractions following
ligand binding.

Thus, using the method of the invention, the
magnetic particles with the TC II-cobalamin complex
attached may be removed onto a suitable surface by
application of a magnetic field, for example, using a
permanent magnet. It is usually sufficient to apply a
magnet to the side of the vessel containing the sample
mixture to cause the particles to congregate at the wall
of the vessel and so isolate them for further analysis.

Especially preferred are superparamagnetic
particles for example those described by Sintef in EP-A-
106873, as magnetic aggregation and clumping of the
particles during the reaction can be avoided. The well-
known magnetic beads made by Bang Particles (US) may be

particularly suited to use in the present invention.

In general, besides the sample under evaluation,
calibration samples with known holo-TC II complex
content will also be assessed in the performance of the
assay method of the invention. Such determinations can
be used to plot a calibration curve from which the TC II
bound cobalamin content of the sample under
investigation may be determined. The nature of the
calibration samples and selection of conversion or
adjustment factors used in the determination of the
holo-TC II complex may vary depending, for example, on
the particular ligands used in the binding and
separation steps of the assay and other aspects of the
method which affect the binding and separation of the
sample, for example, buffer composition, assay
conditions etc. Typically, calibration samples having
holo-TC II contents of 0 to 300 pmol/l will be used.
The reference range within which the value for TC II
bound cobalamin will generally be found is 30 to 160
pmol/l.

The holo-TC II calibration standard may typically
be human, native or recombinant holo-TC II. The use of
holo-TC II as a calibrator in holo-TC II assays is novel
and forms a further aspect of the invention.

Viewed from a yet further aspect, the invention
provides a kit for a diagnostic assay according to the
invention, said kit comprising:

an immobilized or immobilizable specific binding
ligand for TC II or holo-TC II;

preferably a holo-TC II solution of known
concentration and more preferably a set of such
solutions having a range of holo-TC II complex
concentrations;

optionally, a release agent to release cobalamin
from holo-TC II; and

optionally a labelled ligand.

The assay method of the invention determines the

- 27 -

metabolically active pool of cobalamin by determining
the holo-TC II complex or isolating the holo-TC II
complex and then determining the cobalamin associated
therewith and thus provides a convenient method for the
determination of cobalamin deficiency before or after
the onset of clinical manifestations of cobalamin
deficiency. The assay method may be advantageously used
prior to the onset of symptoms since it has an accurate
predictive value of negative cobalamin balance.

Brief Description of The Drawings

The present invention will now be illustrated by
the following non-limiting Examples and the accompanying
drawings in which:

Figure 1 is a standard curve for holo-TC II;

Figure 2 is a plot showing the relationship between
total serum cobalamin and holo-TC II concentration; and

Figure 3 is a plot showing the relationship between
total serum cobalamin and holo-HC concentration.

EXAMPLE 1

Antibodies (e.g. monoclonal or polyclonal
antibodies) specific for TC II are immobilized on
magnetizable particles. An aliquot of serum (100-500
μl) mixed with an equal volume of PBS is allowed to
react for 15 min with an excess of the immobilized
antibody and an excess of a non-overlapping anti-holo-TC
II antibody (e.g. a monoclonal antibody) radiolabelled
with I^{125}. The magnetizable particles are sedimented by
using a strong magnet, the supernatant is removed, and
the particles are washed with PBS.

The radioactivity is measured and the holo-TC II
concentration of the sample is determined by
interpolation on a standard curve.

EXAMPLE 2

Antibodies (e.g. monoclonal or polyclonal
antibodies) specific for TC II are immobilized on
magnetizable particles. An aliquot of serum (600 μl)

mixed with an equal volume of PBS is allowed to react
with the antibody for 30 min. The magnetizable
particles are sedimented by using a strong magnet and
the supernatant removed. The particles are washed once
with PBS and are subsequently treated with sodium
hydroxide (0.3 M) containing potassium cyanide (100 μM),
dithiothreitol (15 mM), and a fixed amount of
cyanocobalamin radiolabelled with I^{125} or Co^{57} in a total
volume of 100 μl for 15 min, to release cobalamin bound
to the immobilized holo-TC II and at the same time
convert all forms of cobalamin to cyanocobalamin and mix
with a fixed amount of labelled cyanocobalamin. A
limited amount of Intrinsic Factor in 100 μl of borate
buffer is added and allowed to react for 10 min. The
magnetizable particles are sedimented by a strong magnet
and 150 μL of the supernatant removed for measuring
radioactivity. The concentration of TC II-bound
cobalamin in the serum sample is determined from a
standard curve.

     Alternatively the released cobalamin may be
measured using Abbot's IMx method or similar methods.

EXAMPLE 3

     Antibodies (e.g. monoclonal or polyclonal
antibodies) specific for holo-TC II are immobilized on
magnetizable particles. To an aliquot of serum (100-500
μL) mixed with an equal volume of PBS is added a fixed
amount of I^{125} labelled holo-TC II and a limited amount
of the immobilized anti-holo-TC II antibody. After
incubation for 15 min the magnetizable particles are
sedimented by using a strong magnet. The particles are
washed with PBS, and the radioactivity measured. The
concentration of holo-TC II is determined from a
standard curve.

EXAMPLE 4

     Recombinant holo-TC II is immobilized on

magnetizable particles. To an aliquot of serum (100-500 μL) mixed with an equal volume of PBS is added a fixed amount of immobilized holo-TC II and an excess of an antibody (e.g. a monoclonal or polyclonal antibody) specific for holo-TC II and radiolabelled with I^{125}. After 15 min incubation the particles are sedimented by using a strong magnet. The particles are washed with PBS, and the radioactivity measured. The concentration of holo-TC II is determined from a standard curve.

EXAMPLE 5

To an aliquot of serum (100-500 μL) is added an excess of biotinylated cobalamin, so as to bind all apo-TC II and apo-HC. After incubation for 10 min, the serum sample is treated with avidin coated magnetic particles for 10 min. The particles are sedimented by using a strong magnet. The supernatant is recovered and mixed with two non-overlapping monoclonal anti-TC II antibodies, one radiolabelled with I^{125} and the other with conjugated biotin. After 10 min avidin-coated magnetizable particles are added and allowed to bind the biotinylated adducts for 10 min. Subsequently, the particles are sedimented with a strong magnet and washed with PBS. The radioactivity is measured and the holo-TC II concentration is determined by interpolation on a standard curve.

EXAMPLE 6

Same as Example 5, but the apo-TC II depleted serum is mixed with a fixed amount I^{125} labelled holo-TC II and a limited amount of anti-TC II antibody immobilized on magnetizable particles. After incubation for 15 min the particles are sedimented by using a strong magnet.

The particles are washed with PBS and the radioactivity is measured.

Example 7

Cobalamin is immobilised onto magnetizable
microspheres, e.g. by covalent coupling or by using
biotin-(Strept)avidin coupling. Typically, Streptavidin
coated magnetizable microspheres are incubated with 1 μM
biotinylated cobalamin for 30 minutes at room
temperature and in the dark. The beads are sedimented
by using a magnet, washed twice with PBS and resuspended
at 1% in PBS + 5 mg/mL HSA. To an aliquot of serum
(100-500 μL) is added the same volume of PBS and 1/10th
volume of cobalamin coated magnetizable microspheres.
The mixture is incubated for 30 minutes at room
temperature in the dark so as to bind all apoTCII and
apoHC. The particles are sedimented by magnet and the
supernate recovered and mixed with two non-overlapping
monoclonal anti-human TC II antibodies, one
radiolabelled with I^{125} and the other conjugated with
biotin. After 10 minutes (Strept) avidin-coated
magnetizable microspheres are added and allowed to bind
the biotinylated adducts for 10 minutes. Subsequently,
the particles are sedimented with a magnet and washed
with PBS. The radioactivity is measured and the holo-TC
II concentration is determined by interpolation on a
standard curve.

Example 8

Same as Example 7 but excess biotinylated cobalamin
is added directly to the serum sample + PBS, so as to
bind all apoTCII and apoHC. After incubation for 10
minutes, the serum sample is treated with (Strept)avidin
coated magnetizable microspheres for 30 minutes at room
temperature, in the dark.

Example 9

M isp¹⋅ (Rabit) antibody specific for human TCII and with an
apparent binding constant >5x10⁹ M⁻¹ was immobilized on 1
μm magnetizable microspheres coated with goat anti-

rabbitIgG antibody (Indica Diagnostics). Serum samples
of 400 μL each from 49 healthy volunteers were mixed
with an equal volume of PBS and 40 μL of the immobilized
antibody (1%). The mixtures were kept at room
temperature in the dark for 1 hour and then microspheres
were sedimented by using a magnet. The precipitates
were washed once with ice-cold wash buffer (PBS plus
0.02% Tween 20) and subsequently resuspended in 50 μL 50
mM dithiothreitol, 0.001% potassium cyanide, and a fixed
amount of 57Co-CN-Cobalamin (Amersham) in phosphate
buffer, pH 7.5. This was allowed to stand for 30
minutes at room temperature after which 25 μL 0.5M
sodium hydroxide was added and 15 minutes later 300 μL
Intrinsic Factor immobilized on Dextran (enough to bind
50% of tracer) in borate buffer, pH 8.6. After 1 hour
at room temperature, in the dark, samples were
centrifuged at 1000g and 4°C for 10 minutes, supernates
carefully removed, and pellets counted in a Packard
Riastar. The concentration of TCII-bound cobalamin was
determined from a standard curve constructed with eight
calibrators (0-500 pM of holoTC-II) treated identically
to samples. 37 serum samples were also analysed with
respect to total serum cobalamin by using the Abbot IMx
B12 assay.

The standard curve for holo TC-II is shown in
Figure 1 and the relation between total serum cobalamin
and holo TC-II concentrations in 37 healthy volunteers
is illustrated in Figure 2. It is evident that the
correlation is low, with r^2 = 0.50. In contrast, the
correlation between holoHC and total serum cobalamin is
high, r^2 = 0.96 (Fig. 3). The average holoTC
concentration for the 49 healthy volunteers was 64±28 pM
and the 95% reference range 23-127 pM.

The assay has a CV of 10%, an analytical
sensitivity (0-calibrator - 3 s.d.) below 10 pM and does
not cross-react with haptocorrin.